STAIN TECHNOLOGY

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A REVISED METHOD FOR THE "QUAD" STAIN

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Since the original publication of this staining method (Stain Techn., 18, 95), various improvements have been made. These are chiefly in the formula for acid alizarine blue, Solution 2, given below and the composition of Solution 4. The author substituted ammonia alum for aluminum sulphate, and Richard C. Webster of our department did the work with the acetic-acid-sodium-acetate buffer. He found that the final staining solution of pH 2.9 gave the sharpest and bluest nuclei and clearest transparent and contrasting cytoplasm of a pinkish hue. With our previous formula, muscle fibrils often stained so deeply that they detracted from the value of the slides, made them too opaque and obscured the nuclei. This has now been overcome. This also allows the elastic tissue to show better and to contrast with muscle fibers. This is especially noted in studies of the heart and blood vessels.

PREPARATION OF SOLUTIONS

Solution 1.	Orcein, synthetic (MacAndrews & Forbes)	0.4 grams
	Nitric acid, C. P. concentrated	0.4 ml.
	Ethyl alcohol, 90%	100.0 ml.
Solution 2.	Acid alizarine blue 2B	0.35 grams
	Aluminum ammonium sulphate C. P. crystals	5.00 grams
	N/10 acetic acid (5.8 ml. glacial acetic acid made up to one liter).	82.00 ml.
	N/10 sodium acetate (13.6 grams C. P. crystals made up to one liter.)	18.0 ml.
	Boil gently in an Erlenmeyer flask covered with a watch crystal for ten minutes. Cool and filter.	
Solution 3.	Orange G. (National Aniline, Certified)	2.00 grams
	Fast green FCF (National Aniline, Certified)	0.20 grams
	Glacial acetic acid	2.00 ml.
	Distilled water	100.00 ml.

Solution 4.	Phosphotungstic acid C. P. crystals	4.0 grams
	Phosphomolybdic acid C. P. crystals	1.0 gram
	Distilled water	100.0 ml.

Solutions 1 to 3 given above last a long time without deterioration. It has been noticed that Solution 1 tends to stain more rapidly after it is several days old and that this should be accompanied by a shortening of the staining period. Solution 1 is best used as a progressive stain.

Method

1. Fix in Zenker or Helly's solution.
2. Embed in paraffin or nitrocellulose or parlodion and section 10μ or less.

3a. Affix nitrocellulose or parlodion sections to the slide as follows: Float section from 95% alcohol onto clean slides and straighten out with a fine brush or drawn-out glass rod. Blot firmly with smooth, hard, blotting paper or bibulous paper, and immediately flow on several drops of 0.25% parlodion in ether-alcohol, allowing excess to run down the slide beyond the section. Hold slide at an acute angle to the eye looking across the film, and when the surface begins to look dull or slightly wrinkled, place immediately in 85% alcohol to harden the parlodion. This is accomplished in about a half-hour. The smaller the amount of parlodion added the better.

3b. Mount paraffin sections on slides as usual and pass through xylene and absolute down to 85% alcohol.

4. Remove any $HgCl_2$ in the sections with iodine in 85% alcohol.
5. Wash out any traces of iodine in 85% alcohol.
6. Stain in orcein (Solution No. 1) 2–24 hours but only long enough to color the elastic fibers a satisfactory red-brown. (If no elastic fibers are in the tissues or if one does not wish to stain elastic fibers, this step may be omitted.)
7. Wash out excess of orcein in 2 changes of 85% alcohol and run slides down to water.
8. Stain in acid alizarin blue 5–10 minutes (Solution No. 2).
9. Rinse in distilled water.
10. Decolorize and mordant in phosphotungstic-phosphomolybdic acid (Solution No. 4) until collagen is destained. (This generally takes 10–30 minutes.) Fresh solution should be made up when the decolorization of the collagen is unduly slow.
11. Rinse a *few seconds only* in distilled water and stain in fast-green-orange-G 10 minutes (Solution No. 3.). If rinse is too long, the mordanting effect of solution 4 is lost.

12. Rinse thoroughly but rather rapidly (about 10 seconds) in 50% alcohol and pass through two changes of 95% alcohol, allowing a few minutes at least for each change. With the parlodion-affixed sections, leave in alcohol until the stain comes out of the parlodion or nitrocellulose surrounding the sections.

13a. Pass parlodion or nitrocellulose sections from 95% alcohol through two changes of terpineol, two changes of xylene and mount in xylene-clarite.

13b. Pass paraffin sections from 95% alcohol through two changes of absolute, two of xylene and mount in xylene-clarite. (60% clarite lumps dissolved in xylene).

Results

Elastic fibers	red-brown
Nuclei, basophilic granules	blue
Cytoplasm and muscle fibers	violet or pink
Collagen, reticulum or basement membranes	green
Erythrocytes, myelin sheaths, acidophilic granules, plasmosomes	orange

SOME STAINING TECHNICS FOR GUAYULE STUDIES

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ABSTRACT.—**For staining tissues and secretions in guayule studies the following dyes and dye combinations have been found satisfactory: rubber in plant sections, Sudan IV; plant sections (rubber and tissues) and ground tissues, Poirrier's blue, Sudan IV and titan yellow; rubber extraction films (with tissue contamination), oil blue NA, safranin O and titan yellow; resin extraction films, Bismark brown and oil blue NA; chopped plants (milling studies), safranin O and Sudan IV. For microscopic studies, sections and ground tissues are pretreated with KOH and a bleaching agent prior to staining.**

INTRODUCTION

The purpose of this report is to record observations made in the course of studies conducted in connection with routine chemical analyses of guayule samples for rubber and resin content. These studies have had to do chiefly with plant fragments; i.e., ground tissues, both before and after analytical extraction, and impurities in rubber films produced in the analyses. Studies have been made on sections of plant stems and roots with the object of learning staining patterns of various structural elements and secretions. For a clear understanding of the following presentation it should be kept in mind that in guayule the rubber-bearing latex does not occur in tubes as in some other plants but is confined in individual cells (Lloyd, 1932).

OBSERVATIONS

In the studies here reported a pretreatment with KOH solution followed by a bleaching agent has resulted in more satisfactory staining of sections and ground tissues than when the dyes are applied without pretreatment. The alkali saponifies suberin and the bleaching solution ("Clorox") seems to promote stain brilliancy. In the case of rubber films, the pretreatment is of no value, and for resin films it is undesirable because of its solvent action on the resin itself.

As a preliminary to sectioning, a short piece of the specimen is soaked in water for 24 hours or up to several months, keeping it at about 5° C. Cut sections are collected in water and kept in the cold until needed. Sections are cut about 50–100μ thick, thus leaving protecting walls above and below the contents of one or two layers of cells. When a cell is cut open the contained rubber is usually

dragged out by the sectioning blade, even when a freezing microtome attachment is used. In the author's experience most guayule sections have rubber agglomerates firmly adhering to their surfaces and causing the sections to stick to one another. If, in lieu of the freezing technic, lengths of carrot be used for holding the material to be sectioned and the carrot sections collected with the guayule sections, the rubber agglomerates are less troublesome.

For staining rubber in guayule and other Compositae, the use of the following dyes has been reported: alkanet (Lloyd, 1911, p. 176), oil blue NA (Addicott, 1944; Whittenberger, 1944), Sudan black B (Haasis, 1944), Sudan III (Artschwager, 1943, p. 3; Artschwager and McGuire, 1943, p. 2; Hall and Goodspeed, 1919, p. 212), and Sudan IV (Haasis, 1944). The first of these has fallen into disuse and is ignored in the current discourse. Of the four others, any one used alone will, for some purposes, give a very acceptable stain, and the choice of one or another is chiefly a matter of expediency or individual choice based upon availability or the stain hue desired. A mixture of Sudan black and Sudan IV stains rubber an intermediate color of no special merit in comparison with the individual dyes.

The colors of unstained guayule tissues afford a certain amount of contrast to stained rubber. There are times, however, when a greater degree of contrast is desirable. Of the dyes commonly used for staining cellulosic, lignified, suberized and cutinized membranes, a number are well suited for use with the rubber-staining dyes. Here, again, the particular dye chosen will depend largely on the contrast effect desired. Oil blue NA and the three Sudan dyes here considered stain suberin much the same as they stain rubber.

As a counterstain for oil blue NA, safranin is excellent (Addicott, 1944) and this can be used to advantage, also, with Sudan black, giving a strong purple-red hue. With Sudan IV, iodine green and methyl green have been used (Haasis, 1944). These afford a good green or blue-green contrast for the crimson of the rubber dye. Bismark brown gives an orange or orange-gray contrast, likewise relatively intense. None of these four dyes stain so intensely as to obscure stained rubber lying upon or enclosed in woody tissues, and even small specks of rubber located in the cork layer can be distinguished fairly well. Another staining effect is obtained by the use of aniline blue W. S. or Poirrier's blue, dyes which have the virtue of coloring sclerenchyma and cork cells more distinctively than do the others just mentioned. Unfortunately, as Conn has indicated (Conn, 1940, p. 135–6), it is not every batch of this type of dye that will give the desired staining effect.

For still more differentiation, yet a third dye can be added. In Addicott's technic, for example, Congo red is used to give a light red hue to the cellulose (Addicott, 1944). For use with Sudan IV and aniline blue, I have found titan yellow eminently satisfactory. In this combination the last-named dye imparts a distinctive yellow or greenish yellow color to cellulose walls of pith, ray, and cortical cells, to walls of epidermal fragments and trichomes. Especially well stained are the walls of the recently formed phloem cells. In strong solution titan yellow stains sclerenchymatized cells as well as those just mentioned, thus producing a green stain when combined with aniline blue. The cork cells, but slightly stained by the yellow dye, appear blue, or blue-gray. The combination of three dyes thus yields four colors and is accordingly superior to the combination of Sudan IV and iodine or methyl green previously recommended (Haasis, 1944), in which sclerenchyma and cork cells are both stained much the same color.

For an analysis of rate of flow in factory processing a solution of safranin O and Sudan IV proved very satisfactory for staining a bucketful of wet guayule chopped to pass through a $1\frac{3}{16}$ inch screen, the stained mass being quite suggestive of loganberry jam. Here the purpose was to stain both rubber and tissues distinctively but without differentiation between the two.

In Table 1 are shown the staining characteristics of various dyes and dye combinations as they have been evidenced in the current studies. The colors as given in this table are to be considered as approximate examples, as the intensity and to some extent the hue vary somewhat with dye concentrations, proportions of solutions in the mixture, and staining times. For the most part observations were made with daylight illumination at a magnification of about 50–100 diameters.

Omission of the pretreatment results in strong staining of the stem cork cells by Sudan IV. In the case of roots, staining of the corresponding external layer is much less intense. With this exception, staining of root tissues is essentially the same as that of stem tissues. It is to be noted that when a cell-wall stain is used in combination with a rubber stain the intensity of rubber coloration will be somewhat dimmed by the color of any overlying cell walls.

Recommended Schedules

On the basis of the observations upon which this report is based a number of recommendations can be made for staining schedules for the several types of guayule material worked with. These are

TABLE 1. COLORATION OF GUAYULE TISSUES AND SECRETIONS BY VARIOUS DYES AND DYE COMBINATIONS[1]

Dye[2]	Solution Proportions[4]	Staining Time hr.[4]	COLORATION[3]						
			Rubber		Resin Extraction Film	Tissues			
			Extraction Film	In and on Sections[5]		Cellulosic	Lignified	Cork Cell Walls	Trichomes
Acid fuchsin	.....	¼	0	0 or fn Pr[6]	.....	0	lt Pr	lt dl Pr	.....
Aniline blue WS[7]	.....	½	0	0	.....	0[8]	Bu	dl Bu	Bu
Bismark brown	19:1[9]	¼	0 or fn Bn	0	Y–Bn[10]	0	Y–Bn	dl Y–Bn	fn Y–Bn
Iodine green[7]	.....	½	0	0[11]	.....	0	Bu–Gn	dl Bu–Gn	.....
Oil blue NA	.....	½	Bu or lt Bu	Bu	lt Bu or pl Bu[12]	0	lt dl Y–Gn	lt dl Bu–Gn	.....
Safranin O	.....	1/60	0	0[13]	.....	0	Pr–R	Pr–R	lt Pr–R
Sudan black B	.....	¼	dl Gy–Bu or dk dl Gy–Bu	dl Bu or dk dl Bu	Gy–Bu or dk Gy-Bu	0	lt dl Y–Gn	lt dl Gn	pl dl Bu
Sudan III	.....	½	Or–R or pl Or–R	Or–R or lt Or–R	lt Or–R or pl Or–R	0	lt Gy or lt Gy–Gn	lt dl Gy–R	0 or fn Or–R
Sudan IV	.....	½	R or lt R	Prsh R or R	R or lt R	0	lt Gy–Gn	lt dl Gy–R	.....

[1]Except as otherwise noted, tissues were pretreated with alkali and oxidant before staining, while rubber and resin films were stained without pretreatment.

[2]Solution formulae are given in Table 3.

[3]Colors are indicated by the following symbols: Bn, brown; Bu, blue; dk, dark; dl, dull; fn, faint; Gn, green; Gy, gray; lt, light; 0, none; Or orange; pl, pale; Pr, purple; R, red; sh, ish; Y, yellow.

[4]For sections; proportions and periods are suggestive, proportion figures indicating drops of solution.

[5]In place in tissues and occurring as superficial agglomerates.

[6]Minute red granules in rubber masses, especially of pith and rays.

[7]Very similar effects are obtained by substituting Poirrier's blue for aniline blue WS or methyl green for iodine green.

[8]Pith cell walls blue and light blue; with prolonged staining, cortical and resin-canal cell walls also become colored.

[9]Water and Bismark brown solution.

[10]In part.

[11]Faint bluish green staining of cell contents, possibly other than rubber, in outer cortex.

[12]Sometimes also blue-green and dull yellow-green.

[13]Light purple-red staining of cell contents, possibly other than rubber, in outer cortex.

Titan yellow	.....	1	0	0	.....	Y	Gnsh Y	lt Gnsh Gy[14]	Y
Oil blue NA and acid fuchsin	8:8	½	.....	Bu	.....	0	Pr–Gy	Gy	.....
Oil blue NA and Bismark brown	15:1	⅓	lt Bu	Bu	lt Gnsh Bu, Y–gn, & dl Bu–Gn[15]	fn Bn[16]	Or–Bn	dl Bn	pl Bn–Gy
Oil blue NA and safranin O and titan yellow	6:1:18[17]	1	lt Bu[17]	Bu	.....	lt Y or pl Y	lt dl Pr & lt dl Or	dl Pr	.....
Sudan black B and safranin O	15:1	⅙	.....	dl Bu or dk dl Bu	.....	0	Pr–R	dk Pr–R	pl dl Bu
Sudan IV and aniline blue WS	8:8[18]	½	.....	Prsh R	.....	0	Bu[18]	Bu–Gy[18]	.....
Sudan IV and aniline blue WS and titan yellow[7]	5:10:15	1	.....	Prsh R	Prsh R or lt Prsh R[19]	Y or Gnsh Y[20]	Bu–Gn, & Ysh Gn	dl Bu–Gy or Dl Gn–Gy	lt Gn or pl Gn[21]
Sudan IV and aniline blue WS and Bismark brown and titan yellow	5:10:1:15	1	.....	Prsh R or lt Prsh R	.....	Y or lt Gnsh Y	Gn	Gy	dl Gn
Sudan IV and iodine green[7]	8:8	½	.....	Prsh R	Prsh R or lt Prsh R	0	Bu–Gn	Bu–Gn	.....
Sudan IV and iodine green; no pretreatment[7]	8:8	1	.....	Prsh R or dl lt Prsh R	.....	dl pl Y–Gn	dl Bu–Gn	dk Pr–R[22]	pl Gy or pl Pr
Unstained	.....	..	fn Gnsh Bn[23]	dl pl Gnsh Y[16]	dl Gn & Bn–Gn	pl Ysh Gy	pl Gnsh Gy[24]	pl Y–Gn[24]	.....

[14]Cork cell walls partially stained yellow.
[15]Mottled.
[16]Or nearly colorless.
[17]For rubber extraction films a 2:3:27 mixture is preferable.
[18]With a ratio of 10:5, the colors are light blue for lignified tissue and light gray for cork cell walls.
[19]A few small particles light blue or light greenish yellow.
[20]In this schedule active phloem is weakly stained in cross sections. More intense staining by titan yellow is afforded by staining one hour in a mixture of 10 parts of aniline blue and 15 parts titan yellow solutions, then adding 5 or 10 parts of Sudan IV solution and staining another ¼ hour. The red stain in this case is a little weaker, however.
[21]Or pedicels pale dull purple.
[22]Outermost dull blue-green.
[23]Due to antioxidant used at one point in the analysis. When the antioxidant is omitted the film color is faint greenish yellow.
[24]Before pretreatment, the sclerenchyma is light gray and the cork layer dull greenish yellow.

listed in Table 2. In Table 3 are shown the solution formulae found satisfactory.

All the schedules shown in Table 2 are carried out at room temperature. Sections are kept in water at about 5° C. until needed and transferred wet to the small vials in which the various operations are to be accomplished. Ground tissues are rinsed on coarse tough filter paper in funnels, or, if not too fine, in Syracuse watch glasses.

TABLE 2. RECOMMENDED STAINING SCHEDULES FOR VARIOUS CLASSES OF GUAYULE MATERIAL*

Aa. *Sections* (for rubber only)

1. Soak in KOH solution ..... 10 min.
2. Add "Clorox" (2 parts to 1 part of above) and soak ..... 5 min.
3. Rinse with water ..... 3 times
4. Add Sudan IV solution and soak ..... 1 hr.
5. Rinse with water ..... 2 times
6. Remove surplus water
7. Mount in "Karo"

Ab. *Sections* (for rubber and accompanying tissues)

1–3. Same as in *Aa*, above .....
4. Add a mixture of titan yellow and Poirrier's blue solutions, in ratio of 15:10, and soak ..... 1 hr.
5. Add Sudan IV solution in an amount equal to that of Poirrier's blue solution, and soak ..... ¼ hr.

6–8. Same as 5–7 of *Aa*, above

B. *Rubber films* (and contaminating tissue fragments)

1. Soak in mixture of solutions of oil blue NA, titan yellow, and safranin O, in the proportion of 2:27:3 ..... 1 hr.
2. Rinse with water ..... 3 times

3, 4. Like 6, 7, of *Aa*, above

C. *Resin films*

1. Soak in mixture of solutions of oil blue NA and Bismark brown, in ratio of 19:1 ..... ¼ hour

2–4. Like 2–4 of *B*, abov

D. *Ground plant tissues*

1. Soak in KOH solution ..... ¼ hr.
2. Add "Clorox" (2 parts to 1 part of above) and soak ..... 10 min.
3. Rinse with water ..... 3 times
4. Add a mixture of titan yellow and Poirrier's blue solutions in the ratio of 16:10, and soak ..... 1 hr.
5. Add Sudan IV solution in an amount equal to half that of Poirrier's blue solution and soak ..... ¼ hr.

6–8. Same as 5–7 of *Aa*, above

E. *Chopped plant tissues*

1. Soak in solution of safranin O and Sudan IV ..... 7 hr.

*Figures given for dye solution proportions represent drops; for KOH and "Clorox", half-milliliters. Solution mixing is performed at the time of using.

TABLE 3. SOLUTION FORMULAE[1]

1.	Acid fuchsin	0.95	g.
	70% ethanol	95	ml.
2.	Aniline blue, water-soluble (certification No. NK-4[2]	0.95	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
3.	Bismark brown Y (Certification No. CN-3[3], dye content 50%)	0.95	g.
	70% ethanol	95	ml.
4.	Potassium hydroxide	10	g.
	95% ethanol	100	ml.
5.	Iodine green	0.095	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
6.	Methyl green (dye content 60%)	0.095	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
7.	Oil blue NA (78235[3])	0.475	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
8.	Poirrier's blue (Lot No. 10268[4])	0.95	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
9.	Chlorox (a commercial bleaching agent containing, according to analysis on label, 5.25% NaOCl and 94.75% inert ingredients)		
10.	Safranin O	0.05	g.
	Water	100	ml.
11.	Safranin O	0.4	g.
	Sudan IV	2.0	g.
	Acetone	2.0	l.
	95% ethanol	1.4	l.
	Water	0.6	l.
12.	Sudan black B (9295[4])	0.095	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
13.	Sudan III (dye content 91%)	0.095	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
14.	Sudan IV (Certification No. NZ 11[2], dye content 86%)	0.095	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.
15.	Titan yellow (practical[5])	0.38	g.
	Acetone	47.5	ml.
	70% ethanol	47.5	ml.

[1]Except as otherwise obvious, all solutions were prepared with distilled water. The dye solutions were filtered before using.

[2]Commission on Standardization of Biological Stains.

[3]Calco Chemical Division, American Cyanamid Co.

[4]National Aniline & Chemical Co.

[5]Eastman Kodak Co.

Citing Romeis, Conn (1940, p. 57), lists an acetone-ethanol solvent for Sudan IV. This mixture has proved very generally satisfactory as a solvent for various dyes in the studies here reported. In this connection Conn mentions an earlier formula of another worker (Herxheimer) in which the solvent is a mixture of ethanol and NaOH. It might be thought that such a combination could be used to saponify suberin simultaneously with the rubber staining. This did not prove to be the case, however.

In conclusion, it may be mentioned that the studies upon which this paper is based have not aimed at an exhaustive exploration of

the possible staining technics, but have been directed, rather, to the development of schedules for use in certain specific lines of investigation. This objective having been attained, further work in the field is not at present contemplated.

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FEULGEN REACTION FOR THYMONUCLEIC ACID[1]

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ABSTRACT.—**The importance of thymonucleic acid in tissues is discussed briefly. The technic of the Feulgen reaction which has been employed in photometric histochemical observations in tumors is described. The evidence for the specificity of the Feulgen reaction is reviewed and additional experimental observations are reported. The staining of tissues by the Feulgen reaction is compared with that of hematoxylin, basic fuchsin, and fuchsin-sulfurous-acid reagent in which the color had been developed by the addition of formaldehyde. The stains were compared with respect to (1) the selective staining of the cytologic components of the tissues, (2) the staining of tissues following varying intervals of acid hydrolysis and (3) the photometric determination of the fading of the stained tissue by a carbon arc light. The photometric apparatus employed is suitable for the study of many problems on the staining of tissues. Staining by the Feulgen reaction is different from that of both the basic fuchsin from which the fuchsin-sulfurous-acid was prepared and from that of the product of the fuchsin-sulfurous-acid which had reacted with an aldehyde. Under carefully controlled conditions, the Feulgen technic is a relatively specific histochemical reaction for thymonucleic acid.**

The Feulgen technic, which was introduced by Feulgen and Rossenbeck in 1924 for the demonstration of thymonucleic acid in tissues, is based upon the Schiff reaction for aldehydes. The Feulgen reaction has been widely used in histochemical observations on plants and animals. As evidence of its widespread usage, Milovidov in 1938 listed 450 references of publications involving this technic. Most investigators feel that under properly controlled conditions the Feulgen reaction is quite specific, but an occasional author questions the specificity. The validity of a considerable amount of research work is based on the presumed specificity of the Feulgen reaction for thymonucleic acid. In this paper the evidence for the specificity of the Feulgen reaction will be reviewed, additional experimental evidence will be presented and comments will be made on the basis of the author's experience with the use of the technic in the photometric determination of thymonucleic acid in tumors.

[1]This investigation was aided by a grant from the International Cancer Research Foundation.

Thymonucleic Acid

To understand the value of the Feulgen reaction one must first appreciate the importance of thymonucleic acid. Nucleic acids seem to be virtually universal constituents of living material which is capable of self-reproduction. They have been demonstrated in a wide variety of animal and plant tissues, in protozoa, bacteria and filterable viruses. Nucleic acid of the thymus type is found chiefly in the chromatin, and nucleic acid of the yeast type in the cytoplasm and nucleoli of cells. The former type of nucleic acid has been called thymonucleic acid, zoonucleic acid, chromonucleic acid, desoxypentose nucleic acid, desoxyribose nucleic acid or *d*-ribodesose nucleic acid. The latter type of nucleic acid has been termed yeast nucleic acid, phytonucleic acid, plasmonucleic acid, pentose nucleic acid or *d*-ribose or ribose nucleic acid. Although the terminology has been recently discussed by Pollister and Mirsky (1943, 1944) and Gulland, Barker and Jordan (1944), the commonly used biological terms thymonucleic and yeast acid seem satisfactory in view of the limited state of our present knowledge regarding the nucleic acids.

Thymonucleic or desoxyribonucleic acid is one of the important constituents of the chromatin of cells. In combination with proteins, supposedly of the histone or proteose type (Caspersson 1940b, Schultz 1941), it forms the nucleoprotein of the genes. The role of thymonucleic acid in normal cells has been discussed by Caspersson (1939), Schultz (1941, 1944), Mirsky (1943) and Mirsky and Pollister (1943) and Greenstein (1944). The evidence that some tumors show a disturbance of nucleoproteins and that roentgen radiation causes an imbalance of nucleic acids has been recently reviewed by Stowell (1945a,b).

The chemistry of the nucleic acids has been discussed in the monograph by Levene and Bass (1931) and the literature on nucleoproteins has been recently reviewed by Greenstein (1944). Chemical formulae for thymonucleic acid have been given by Levene and Bass (1931), Gulick (1941) and Greenstein (1944). Although the exact chemical configuration of the molecule may not be definitely known, it seems certain that the thymonucleic acid contains adenine, guanine, cytosine, thymine, *d*-ribodesose and phosphoric acid. Ribonucleic acid differs from desoxyribonucleic acid in that it contains the carbohydrate *d*-ribose instead of *d*-ribodesose and uracil instead of the methylated pyrmidine, thymine. On the basis of these differences in the carbohydrate one may distinguish the two types of nucleic acids.

Since the yield of thymonucleic acid by the present methods of extraction is not highly quantitative, one may resort to chemical determination of nucleic phosphorus, purines, purine nitrogen, to color reactions of desoxyribose or to measurement by ultraviolet absorption. Determinations of thymonucleic acid by the color reactions or by ultraviolet absorption are relatively accurate and have been adapted for observations on intact tissues. The cytochemical methods of studying thymonucleic acid in tissues by ultraviolet absorption have been developed chiefly by Caspersson (1940a, b). There are six chemical tests for 2-desoxypentose sugars. The methods of Kiliani (1913), Thomas (1931), the pine shaving test, and the two methods of Dische (1930, 1944) have not been adapted for observations on sections of tissue. Desoxyribose sugars react with fuchsin-sulfurous-acid according to the Schiff reaction, like aldehydes, to produce a reddish-purple pigment. This color reaction is the basis for the Feulgen technic of demonstrating thymonucleic acid in stained sections of tissue.

Feulgen Reaction

The Feulgen reaction is carried out in two steps. The first step is a mild acid hydrolysis, which splits the purine bases and the carbohydrate of the thymonucleic acid, and liberates the aldehyde groups of the aldo-pentose sugars. The second step is a reaction between the liberated aldehyde groups and the fuchsin-sulfurous-acid reagent (leuco basic fuchsin). In the formation of this reagent, basic fuchsin is reduced and decolorized by the action of sulfur dioxide. After immersing the partially hydrolyzed tissue in this fuchsin-sulfurous reagent, the portions of nuclei containing thymonucleic acid stain an intense reddish-purple. The details of the reaction have been discussed by Feulgen (1932) and de Tomasi (1936).

Several suggestions for slight modifications in the Feulgen reaction have improved the technic and its specificity. Feulgen originally stated that sublimate-acetic fixative should be employed. He warned against the use of solutions containing chromic acid which might destroy the nuclear material and of formaldehyde which might give a positive color reaction. The work of Bauer (1933) and Hillary (1939) has shown that virtually any fixative may be used if the tissues are washed properly. To overcome the irregularities produced by unsatisfactory basic fuchsin, de Tomasi (1936) and Scanlan and Melin (1937) have suggested the use of a specially prepared pararosaniline dye. Coleman (1938), however, found a simple and more efficient method of preventing the staining of the cytoplasm

by the impurities of the staining solution. By treating the bleached solution with activated charcoal, which removes the extraneous coloring matter, a colorless water-clear, fuchsin-sulfurous reagent can be obtained from any sample of basic fuchsin. The use of potassium metabisulfite instead of sodium sulfite as a source of sulfur dioxide in fuchsin-sulfurous reagent, as suggested by de Tomasi (1936), gives a more dependable and stable solution.

The exact technic employed by the author in his observations on thymonucleic acid in tumors (Stowell 1942, 1945a,c) has not been published previously. The Feulgen technic has been slightly modified by changing the acid hydrolysis to give precise and uniform results which are satisfactory for photometric measurements. Because of the use of quantitative photometric methods, no counterstain was employed. Tissues were fixed in equal parts of 95% ethyl alcohol and a saturated solution of $HgCl_2$, as suggested by Cowdry (1928). The fuchsin-sulfurous reagent was prepared according to the following directions which are essentially the same as those of de Tomasi (1936) with the incorporation of suggestions by Coleman (1938).

1. Dissolve 0.5 g. of basic fuchsin by pouring over it 100 ml. of boiled distilled water. Shake thoroughly. Cool to 50° C. and filter.

2. Add 10 ml. of normal HCl and 1 g. of potassium metabisulfite ($K_2S_2O_5$) to the filtrate. Shake, close tightly in a chemically clean bottle, and store in the dark for 24 hours.

3. Add 0.25 g. of neutral activated charcoal; shake for 1 minute and filter rapidly through coarse filter paper. The clear filtrate is stored in the dark.

The basic fuchsin employed was a product of the National Aniline and Chemical Company, with the Stain Commission certification number of NF 33. The activated bone charcoal was obtained from Merck and Company. For the staining of each group of slides, a fresh staining solution was prepared twenty-four to forty-eight hours before use.

The standard technic for the staining of paraffin sections of tissues was as follows:

1. Remove paraffin in xylene; leave 24 hours in 95% ethyl alcohol (to remove lipids which might cause plasmal reaction); and then hydrate to water.

2. Rinse in cold normal HCl for 1 minute.

3. Hydrolyze in normal HCl at 50° C. for 20 minutes. (Although this permits more precise control, the more rapid hydrolysis for 5 minutes at 60°C. is satisfactory for routine purposes).

4. Rinse in cold normal HCl for 1 minute.
5. Stain in fuchsin-sulfurous-acid reagent for 2 hours.
6. Drain and pass quickly to the first of 3 closed Coplin jars containing the acid bleaching solution. Leave 10 minutes in each jar.
7. Rinse in tap water for 5 minutes.
8. Rinse in distilled water.
9. Dehydrate and mount in Clarite.

Control sections are treated in the same manner except that steps 2 to 4 are omitted to eliminate the hydrolysis of the thymonucleoproteins.

The sulfurous acid bleaching solution contained 5 ml. of normal HCl, 5 ml. of 10% potassium metabisulfite ($K_2S_2O_5$) and 100 ml. of distilled water. A 60% solution of clarite in xylene was employed for mounting sections because it is absolutely neutral according to Groat (1939) and does not promote fading of most stains as much as neutral balsam (Lillie, 1941; Stowell, unpublished observations). Also, it has been shown by Stowell and Albers (1943) to absorb less visible light than balsam. Precautions such as storage in the dark, were taken to reduce any tendency toward fading of the stain, although Hillary (1939) claims that fading does not occur in neutral mounting media. Photometric measurements on material used in other problems as well as the observations to be described in this paper have not produced evidence of appreciable fading of the Feulgen-stained material.

The exact chemistry of the Feulgen reaction is not clearly understood. Wieland and Schening (1921) have suggested that the aldehydes react with the colorless fuchsin-sulfurous reagent to form a colored addition compound. Baker (1942) gives structural formulae of the colorless and regenerated fuchsin-sulfurous-acid and attributes the color to the reformation of the quinonoid linkage.

The spectrophotometric analyses of Stowell and Albers (1943) have furnished some valuable information on the relation of the reddish-purple pigment of the stained thymonucleic acid and the color of basic fuchsin. Spectrophotometric absorption curves were determined for three different solutions. Measurements were made on a 0.5% aqueous solution of basic fuchsin containing 10% of normal HCl. The stain was then converted to the colorless leuco form by the action of potassium metabisulfite. After the addition of 2 drops of 40% formaldehyde to 10 ml. of the solution, as in the Schiff reaction, absorption measurements were again made. The third set of absorption measurements were made on a section of thymus gland of a rabbit which had been stained by the Feulgen technic.

The absorption curves definitely indicated that aqueous basic fuchsin represents a different dye from the fuchsin-sulfurous-acid reagent after treatment with formaldehyde or the staining of thymonucleic acid in thymus. The product of the Schiff reaction had absorption coefficients 150 times those of the basic fuchsin stain, had its maximum absorption at 550 instead of 560–570 mμ, and did not show the secondary absorption band at 400 mμ. Since in the Schiff reaction and Feulgen technic the basic fuchsin is reduced by sulfurous acid to form the fuchsin-sulfurous-acid reagent, one should not expect the final stain to be the same as the original basic fuchsin. Although some people have assumed that the aldehydes produced a regeneration of the original pigment, Feulgen and Rossenbeck (1924) recognized that the final product was more blue than the original basic fuchsin. Inspection of the absorption curves obtained by Stowell and Albers shows that this increased blue is caused by a relatively decreased absorption of shorter wave lengths rather than by an increased absorption of the longer red wave lengths of light.

The Specificity of the Feulgen Reaction

The specificity of the Feulgen nucleal reaction has been questioned by some authors largely on the basis of theoretical reasoning and supported by other men on the basis of their actual experience. In a series of papers Feulgen and his co-workers, Rossenbeck, Voit and Imhäuser (for bibliography see Feulgen, 1932), tested the Feulgen reaction on many tissues and laid the foundation for the claims of specificity of the reaction. They showed that the staining is specific for the potential aldehyde groups of thymonucleic acid and that after hydrolysis these groups remain localized instead of diffusing throughout the cell.

Semmens (1940) noted that certain purines such as pyridine, piperidine, caffeine, theobromine, adenine and guanine gave a positive test with fuchsin-sulfurous-acid reagent. He suggested that the Feulgen reaction may be produced by the purine constituents of nucleoproteins rather than by the carbohydrate component. Barber and Price (1940), however, promptly pointed out that such effects are not chemically equivalent to the Feulgen reaction, but are simply due to the basicity of the purines, the effect of which was pointed out by Feulgen and Rossenbeck in 1924. Barber and Price concluded, as have Feulgen (1932), Baker (1942), Mirsky (1943) and many others, that the reaction is specific for the potential aldehyde groups of chromatin.

That sugars or certain other substances besides desoxyribose might

give a positive Feulgen reaction has been suggested, but the instances in which such reactions actually occur are few. Hillary (1939) found that lignin, suberin, cutin, starch, glycogen, and other polysaccharides may stain with the fuchsin-sulfurous reagent. Such reactions will occur without hydrolysis in control sections and represent a Schiff's test for the aldehyde groups. Certain lipid complexes occasionally found in the cytoplasm may give positive reactions with fuchsin-sulfurous reagent. These plasmal reactions are avoided by leaving the sections in 95% ethyl alcohol. The occurrence of such reactions, which can be detected by the use of unhydrolyzed control sections, are rare in actual practice. Most carbohydrates are readily soluble in aqueous fixing fluids and are removed before staining occurs.

The most recent criticism of the specificity of the Feulgen reaction for thymonucleic acid has been raised by Stedman and Stedman (1943a, b and 1944) and by Choudhuri (1943). Stedman and Stedman have reported the discovery of a new protein "chromosomin" which they claim is the most important constituent of chromatin. They claim that, although thymonucleic acid may produce the Feulgen reaction within the nucleus, the stain produced is water soluble and diffuses throughout the nucleus to stain the chromosomin. Since the details of the methods employed by Stedman and Stedman have not yet been published, their results cannot be critically evaluated at this time. They have criticized the methods and results of other investigators, in some instances without having consulted the original articles, and have suggested several alternative theories for which there is little if any experimental evidence. Although Stedman and Stedman admit that some thymonucleic acid may be present in the chromosomes, they believe that it is a more important constituent of the nuclear sap. In refuting this concept, Callan (1943), Barber and Callan (1944) and Caspersson (1944) review the generally accepted evidence that thymonucleic acid is localized within the chromatin.

A part of the evidence of Stedman and Stedman (1943a, 1944) that the chromatin is composed of chromosomin is based on the similarity of the staining reactions of the chromatin and of the extracted chromosomin with methylene blue, gentian violet, hematoxylin and reduced fuchsin-sulfurous acid (Feulgen) reagent. However, it is doubtful whether the staining of chromatin by these dyes is sufficiently typical to justify the inference that any substances which stain similarly are necessarily identical. Dyes such as hematoxylin change their color depending upon several factors including the hydrogen

ion concentration, hence the observation of Stedman and Stedman (1943a), that an acidic protein such as casein might stain more purple than a basic protein, is not surprising.

Claude and Potter (1943) isolated chromatin threads of leukemic blood cells and showed that they contained 40% thymonucleic acid and stained with the Feulgen reaction. This observation shows that the thymonucleic acid is present in the chromosomes and does not support the suggestion of Stedman and Stedman that fuchsin-sulfurous-acid reagent reacts with thymonucleic acid in the nuclear sap and then stains the chromosomes. Feulgen and Rossenbeck (1924) showed that hydrolyzed thymonucleic acid does not diffuse throughout the cell. Material may remain in water many hours or even days without influencing the subsequent staining by the fuchsin-sulfurous-acid reagent.

That the Feulgen reaction stains the thymonucleic acid attached to the chromosomes is also shown by the observations of Mazia and Jaeger (1939). These investigators digested the cells of the salivary gland of *Drosophila* with a nuclease which splits nucleoproteins. They then stained the tissue for thymonucleic acid by the Feulgen reaction. In the control preparations the chromosomes were stained and the cytoplasm was unstained. In the specimens treated with nuclease the chromosomes were unstained and the cytoplasm stained. The protein of the chromosomes remained intact after treatment with nuclease. These results agree with the conception that the staining reaction in the properly controlled Feulgen reaction is localized in the thymonucleic acid of the chromosomes, and that only after the splitting of the nucleic acids by nuclease does one get a diffusion of the hydrolysis products and a non-specific staining reaction.

In conjunction with the claims of Stedman and Stedman, Choudhuri (1943) suggested that one may obtain the same results as those of the fuchsin-sulfurous-acid reagent of Feulgen by staining tissues with fuchsin-sulfurous-acid reagent in which the color has been developed by the addition of a trace of aldehyde. That such treatment of tissues by developed fuchsin-sulfurous-acid reagent produces different staining results from those of the Feulgen technic will be shown in the following experiments. The fact that the controlled acid hydrolysis splits the thymonucleic acid before completely destroying the protein of the chromatin was confirmed by an hydrolysis experiment in which different stains were employed. Comparable sections of a transplantable mammary carcinoma of a rat (Stowell, 1945a) were hydrolyzed in normal HCl at 60°C. for intervals up to two and a half hours. At intervals of a half hour

sections were removed and stained (1) by the Feulgen reaction, (2) with fuchsin-sulfurous-acid to which a trace of formaldehyde had been added and (3) with Harris' hematoxylin.

After an hydrolysis of one half hour the nuclear chromatin stained faintly with the Feulgen reaction, while after an hour only an occasional nucleus showed any trace of the stain. After one and a half hour's hydrolysis no material reacted with the fuchsin-sulfurous-acid reagent. After an acid hydrolysis of a half hour or of an hour, the slides which were stained with the fuchsin-sulfurous reagent containing a trace of aldehyde showed a rather faint and diffuse nuclear staining. After longer periods of hydrolysis the tissues still stained, but there was little differentiation between nucleus and cytoplasm. The tissues stained with Harris' hematoxylin showed a progressive tendency to decreasing intensity and precision of nuclear staining as the length of time of acid hydrolysis was increased. After two and a half hours hydrolysis the nuclear staining was still perceptible. Thus, as determined by their staining reaction with the hydrolyzed tissue, all three methods of staining produced different results and are apparently dependent upon different conditions within the tissues. These observations present additional evidence that the Feulgen reaction fails to develop after complete hydrolysis of the thymonucleic acid, whereas the remaining nuclear protein still absorbs stain. The staining mechanism of fuchsin-sulfurous-acid reagent and of developed fuchsin-sulfurous-acid reagent containing aldehyde seemed to be different.

When sections stained by the Feulgen reaction and by developed fuchsin-sulfurous reagent are placed in 95% alcohol or in distilled water, the nuclear chromatin of neither preparation is appreciably destained after 48 hours.

To compare the staining of tissues by the Feulgen reaction, the developed fuchsin-sulfurous-acid reagent, basic fuchsin and hematoxylin, a series of comparable slides of biopsies of an epidermoid carcinoma of the skin were prepared. Some sections were stained by the routine Feulgen technic. Other sections were left for one minute in a solution of fuchsin-sulfurous-acid reagent in which the color had been developed by the addition of a drop of dilute formaldehyde. These sections were then dehydrated and destained in alcohol. Similarly sections were stained for one minute in a 1% aqueous solution of basic fuchsin and destained in alcohol. The same dry basic fuchsin was employed in the preparation of all of the stains. Other sections were stained with Harris' hematoxylin. At least two adjacent serial sections were stained by each method and

mounted in Clarite so that one section could be utilized as a control for subsequent experiments on the fading of the stains.

The hematoxylin and basic fuchsin both stained the nucleoli intensely. With the fuchsin-sulfurous-acid reagent developed by the addition of formaldehyde, the nucleoli were stained somewhat less distinctly. After treatment with the Feulgen reaction, only an occasional nucleolus was faintly perceptible. The keratin stained with both basic fuchsin and developed fuchsin-sulfurous-acid reagent, but certain connective tissue elements were intensely stained only by basic fuchsin. Only nuclear material was sharply stained by the Feulgen reagent. Thus the staining with developed fuchsin-sulfurous-acid, as suggested by Choudhuri (1943), gives results which are different from either staining by the Feulgen reaction or basic fuchsin, but more like those of the basic fuchsin.

The rate of fading of the stained sections was compared following two hours exposure to the unfiltered light of a carbon arc. The extent of the fading was determined by measurements in a special microphotometric instrument which consists of a stable light source, light filters, a microscope, a photocell and recording apparatus. This apparatus, which is suited for the measurement of pigment in sections or smears of tissues, has been described briefly elsewhere (Stowell, 1942). Such apparatus has many possible uses in research on the staining of tissues by the determination of the relative intensity of the stain in tissues treated by different procedures. In these sections the mean percentage absorption of light was determined for 25 areas in each control and light-exposed section. The results of these measurements and their statistical significance are shown in Table 1. Each section exposed to the light of the carbon arc absorbed less light within the photometric apparatus than the corresponding unfaded control slide. As shown by the values of P in table 1, this difference, however, was statistically significant (*i.e.*, $P<0.01$) only for tissues stained by the fuchsin-sulfurous reagent containing formaldehyde and by the basic fuchsin. From these observations it is evident that the pigment formed within the tissues by the Feulgen reaction is more stable and resistant to alterations by the physical agent light than the pigment of tissues stained by the developed fuchsin-sulfurous-acid reagent or basic fuchsin. Thus, the staining technic suggested by Choudhuri (1943) by which chromosomes are stained by the reduced fuchsin-sulfurous-acid-reagent of Feulgen represents a staining reaction with a basic dye which differs in several respects from that of either basic fuchsin or the Feulgen reaction.

There is an increasing amount of evidence suggesting that many

if not all staining processes represent an adsorption phenomena (Stowell and Albers, 1943). Silver (1942) has shown that silver staining is an adsorption phenomenon, and Owens and Bensley (1929) believed that staining with osmic acid also represents an adsorption process in which the osmic acid is first reduced by unsaturated compounds and then selectively adsorbed. The staining solutions measured by Stowell and Albers (1943) had the same absorption spectrum as the tissues with which they had been stained. These spectrophotometric measurements showed that the pigment of fuchsin-sulfurous-acid reagent treated with formaldehyde and of thymus stained with the same reagent had similar absorption curves.

TABLE 1. COMPARISON OF FADING OF STAINED TISSUES BY LIGHT.

Stain	Percentage fading*	P†
Harris hematoxylin	8	.1075
Feulgen reaction	12	.2843
Fuchsin-sulfurous acid reagent and formaldehyde	33	.0001
Basic fuchsin	41	.0000

*The percentage fading was calculated from the formula $100 - \left(\frac{A_1 \times 100}{A_2} \right)$ in which A_1 and A_2 represent the mean relative absorption of 25 areas of the section exposed to light and the control section respectively.

†The probability, P, is determined according to the formula given by Rider (1939, p. 80). Values of P which are less than .01 indicate that there is less than one chance in a hundred of obtaining differences of the same magnitude by chance and are considered statistically significant.

The reported observations on the Feulgen reaction would be consistent with a staining mechanism in which there is a focal microchemical reaction between the fuchsin-sulfurous-acid reagent and the aldehyde groups of the hydrolyzed thymonucleic acid. These aldehyde groups are adsorbed to the remaining unhydrolyzed parts of the thymonucleoprotein molecules which form the protein framework of the chromosome. Under the carefully controlled conditions of the Feulgen reaction there is apparently an insignificant rate of diffusion of the split products of the thymonucleoprotein or of the final stained addition compound of the fuchsin-sulfurous-acid reagent and aldehyde radicals. Such a theory of the staining mechanism of the Feulgen reaction may be difficult to establish more conclusively until we obtain a better fundamental knowledge of the molecular reactions of nucleoproteins and dyes.

The extensive work of Caspersson and his co-workers (1939, 1940b,

1944), as well as the few observations of Wyckoff, Ebeling and Ter Louw (1932), show that ultraviolet studies agree with the Feulgen reaction in the localization of the thymonucleic acid within the cells. Although the possibility of false positive tests may be admitted in theory, their presence in actual practice is rare. The available information indicates that the Feulgen reaction for thymonucleic acid is one of the most specific histochemical reactions.

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HISTOLOGICAL METHODS FOR THE DEMONSTRATION OF GOLD IN TISSUES

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ABSTRACT.—**Two methods for the demonstration of gold in tissues are described. The tissue is fixed in neutral formalin, embedded in paraffin, sectioned and run down to water. In the $SnCl_2$ method, modified from that of Christeller, it is then incubated for 24 hours at 56° C. in a mixture of ten parts of 5% $SnCl_2 \cdot 2H_2O$ and one part of concentrated HCl. The interpretation of the results obtained by this method is frequently difficult because of the presence of accessory precipitates and the presence of the normal pigments of the tissue. This has led to the development of a new method, in which the sections are incubated for periods varying from 24 hours to 6 days at 37° C. in 3% H_2O_2. The gold is reduced to the metallic state, the interfering tissue pigments are bleached, and, since no metallic ions have been added, accessory precipitates do not occur. After both methods, the sections are washed thoroughly, run up, and mounted in damar.**

The accumulation of gold in the tissues after the administration of various gold compounds has stimulated the development of methods for determining the distribution of gold by histological procedures. The demonstration of the existence of the gold by microchemical assay of extracts or by spectrographic analysis still leaves unanswered the important question of localization within the tissues.

Of the older histological methods, the one which, in our experience, has proven most reliable is the use of $SnCl_2$. This method was adapted from the technics of inorganic chemical analysis by a series of investigators, including Christeller (1927), Korosu (1927), Okkels (1929) and Michaelis (1930). Although it has been assumed that this method results in a complex of tin and gold, the predominant effect may be simply reduction of the gold.

We have used the $SnCl_2$ method with satisfactory results on tissues of rats and guinea pigs after intraperitoneal injections of yellow gold chloride in aqueous solution. The tissues are fixed by injection of neutral formalin through the heart. After the usual procedures incident to the cutting of paraffin sections, the paraffin is removed and the sections run down to water. A stock 5% aqueous solution of $SnCl_2 \cdot 2H_2O$ is prepared, with some pieces of metallic tin added to retard the oxidation to $SnCl_4$. The slides are placed in a

mixture of ten parts of the stock $SnCl_2$ solution and one part of concentrated HCl, prepared and filtered just before use. This reagent is allowed to act in a 56°C. incubator for 24 hours. Sections left in the incubator for 48 hours show no appreciable improvement. The incubation is followed by washing several times in distilled water before running up through the alcohols and mounting in damar.

The presence of gold, after $SnCl_2$ treatment, is indicated typically by the presence of particles exhibiting the purple of Cassius, grading into brown. The red, blue and black of colloidal gold may also be present.

Two disadvantages of the $SnCl_2$ method have led to the development of a new method for the demonstration of gold in tissues. The first disadvanatge is the occasional presence of precipitates of tin unrelated to the gold. This difficulty is inherent in any method involving the addition of metallic ions, and is even more severe in the $AgNO_3$ methods of Borchardt (1928) and Roberts (1935). The second disadvantage lies in the difficulty of distinguishing the gold deposits made visible by the $SnCl_2$ method from pigments, such as bile pigments, which may be present in the tissue.

These disadvantages are avoided by the new method which we have developed. After neutral formalin fixation and paraffin embedding, the sections are de-paraffined and run down to water. They are then placed in 3% H_2O_2 in a 37°C. incubator for at least 24 hours. With most tissues satisfactory results are obtained with incubation for 3 days, although more complete reduction may be obtained by longer treatment. After washing in distilled water, the sections are run up and mounted in damar.

The gold in the tissues is reduced to the metallic form by the peroxide and displays the range of colors of colloidal gold, with rose predominating, grading into purple, blue and black. The peroxide bleaches the interfering pigments, consequently removing one of the disadvantages of the $SnCl_2$ method. Since no metallic ions have been added, the confusion arising from incidental deposits of metal is eliminated.

It is possible to vary the details of the method by using higher concentrations of H_2O_2, but we have found the commercial 3% solution satisfactory. Although theoretically the reduction of the gold should proceed more rapidly if the solution is alkaline, this effect is more than counterbalanced by the increased tendency of the sections to separate from the slides under alkaline conditions. Even with the peroxide and $SnCl_2$ methods outlined above, we have found

it advisable to increase the affixation of the sections by placing them in formaldehyde vapor for one hour after mounting them with egg albumen.

For critical evaluation of the presence of gold, the sections should be mounted without staining. The identification of the particular tissue constituents involved is facilitated by staining. Hematoxylin

FIG. 1. Photomicrograph of rat kidney prepared by the H_2O_2 method and stained with hematoxylin and light green SF yellowish. The gold deposits in the convoluted tubules appear as dark granules.

interferes only slightly with the identification of the gold. Due to the range of color of colloidal gold, it is difficult to find a satisfactory cytoplasmic stain which will not mask them. When the deposits of gold are sufficiently dense, eosin is satisfactory; under other circumstances light green S. F. yellowish is less likely to mask the color.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

GERSHENFELD, LOUIS. **Bacteriology and Allied Subjects.** 7 by 10 inches. 562 pp. Cloth. Mack Publ. Co., Easton, Pa. Price, $6.00.

This book lies rather outside the field of those usually reviewed in this journal. Attention should be called, however, to the chapter on Stains and Staining, which presents in brief form certain practical information about the chemistry of dyes as well as the most common bacteriological staining formulae. The author has been very careful to make all these staining formulae conform with the standards followed by the Biological Stain Commission.—*H. J. Conn.*

KOLMER, J. A. and BOERNER, FRED. **Approved Laboratory Technic.** *Fourth Edition.* 6½ by 9½ inches. 1018 pp. Cloth. D. Appleton-Century Co., New York. Price $10.00.

This book, which is the outgrowth of the much smaller first edition prepared under the auspices of the American Society of Clinical Pathologists, has for years been a standard text in clinical laboratories, and indeed in many other laboratories where microscopic and diagnostic methods are used. The new edition is more than 100 pages longer than the preceding; it has been largely rewritten and the material rearranged. Because of the rearrangement, it takes more than a cursory examination of the book to discover what new material has been added and how extensive the changes have been. Apparently the authors have taken all pains to cover the field as completely and accurately as possible.

As an illustration of the changes effected one can point out an instance which occurs in a field specially interesting to many readers of STAIN TECHNOLOGY. In the Third Edition, the section on Methods of Examination of Malaria occupied about four pages of text with no illustrations; in the Fourth Edition, about one page of text has been added, three pages of splendid color illustrations which, together with three pages of explanations of the plates, bring the total pages of the section up to eleven. It is felt that these color plates should be very useful in the diagnostic laboratory. Although this section is really but a very small part of the entire book, the changes made in it serve well as an illustration of how the new edition of the book compares with the preceding.—*H. J. Conn.*

MICROSCOPES AND OTHER APPARATUS

CONGER, P. S. **A simple method for micrometric ruling, and some applications.** *Trans. Amer. Micr. Soc.*, **63**, 358–61. 1944.

A crystal of silicon-carbide, carborundum, or a black diamond is mounted with sealing wax or dental impression compound on a micromanipulator or other rigid lever which is regulated by two sets of screws. This is attached to the front of the microscope with the crystal in the field of vision and serves as a cutting tool. The mechanical stage of the microscope forms the ruling engine. As many as 30,000 lines per inch can be made without difficulty.— *V. Kavanagh.*

MICROTECHNIC IN GENERAL

BURFITT, N. E. **A technique for the cutting of frozen sections with the Cambridge rocking microtome.** *J. Path. & Bact.*, **56**, 443–5. 1944.

By means of a special holder for tissue and CO_2 nozzle, frozen sections may be cut on the rocking microtome.—*S. H. Hutner.*

JACOBY, F. **A method of obtaining permanent preparations for the cytological study of pure cultures of macrophages, with special reference to their mode of division.** *J. Path. & Bact.*, **56**, 438–40. 1944.

Permanent preparations of tissue cultures maintained in Carrel flasks can be made without sacrifice of the flask, and manipulations are facilitated by the following device. A Carrel flask about 5 cm. in diameter has a hole of 30 mm. diameter bored in its upper side. A culture on a cover slip may be inserted through this hole, and the top hole may then be filled with a mica plate sealed to the flask with paraffin.—*S. H. Hutner.*

MACINTOSH, O. C. **Purification of paraffin.** *J. Techn. Methods* and *Bull. Internat. Assoc. Med. Museums*, **22**, 99–100. 1942.

The author describes a method for the purification of paraffin which has been used in the embedding of tissues. The procedure is as follows: hold 1000 ml. of the paraffin to be reclaimed overnight in an oven at 56° C. in an open flat container; then heat 10 min. at 75° C. over a bunsen burner; add 25 ml. commercial Fuller's earth and mix well for 5 min. at 75° C.; filter at 100° C. using a heated funnel of the hot water jacket type. Ordinary filter paper is satisfactory.

This technic may be applied to the processing of "Parowax" and solid hydrocarbon waxes as well as commercial paraffin. Because xylene boils at 144° C., it is advisable to use chloroform in the preparation of the sections when the wax is to be reconstituted.—*C. Randall.*

DYES AND THEIR BIOLOGICAL USES

BERNSTEIN, H. I., and ROTHENSTEIN, L. R. **Phenothiazine chemistry. I. 10-sulfanilylphenothiazine and other 10- substituted phenothiazine derivatives.** *J. Amer. Chem. Soc.*, **66**, 1886. 1944.

In connection with the search for antimalarials, certain 10-substituted phenothiazines were prepared for testing because of the structural similarities existing between atabrine, p,p′-bis-(acelylamino)-diphenylsulfone, methylene blue, phenothiazine and phenothiazine sulfone. The first three of these compounds have been found to possess antimalarial activity. The pharmacological findings on the compounds prepared will be reported later.—*A. P. Bradshaw.*

CROMWELL, N. H., and HOEKSEMA, HERMAN. **α, β-Diamino ketones. III. Reaction of 8-amino-6-methoxyquinoline with α-bromo-β-aminoketones.** *J. Amer. Chem. Soc.*, **67**, 124. 1945.

Five new mixed diamino ketones containing the 8-amino-6-methoxyquinoline group have been prepared for antimalarial testing and the results will be published elsewhere. Preliminary pharmacological "screening" tests (i.e. pressor, analgesic, anticonvulsant, etc.) have shown no outstanding effects.—*A. P. Bradshaw.*

DION, W. M. and LORD, K. A. **A comparison of the toxicity of certain dyestuffs to the conidia of Fusarium culmorum.** *Ann. Appl. Biol.*, **31**, 221–31. 1944.

The toxic action of a number of dyes to fungus spores was determined to learn whether there was any correlation between toxicity and chemical structure. Toxicity was tested by the slide-germination technic; *Fusarium culmorum*, and to a lesser extent *Cercosporella herpotrichoides* were the organisms used. Solutions of the following dyes were used in this study: malachite green, tetramethyl-*p*-diamino-triphenyl carbinol; brilliant green, tetraethyl-*p*-diamino-triphenyl carbinol (sulfate); tetra-*n*-propyl-*p*-diamino-triphenyl carbinol (chloride); tetra-*n*-butyl-*p*-diamino-triphenyl carbinol (chloride); aniline hydrochloride; *p*-amino-benzyl alcohol; dimethyl-*p*-toluidine; Mischler's hydrol (base, chloride); auramine (base, chloride); dimethyl-*p*-amino-triphenyl carbinol (chloride); leuco-malachite green (chloride); magenta (base, chloride, sulfate); magenta (chloride); methyl violet 2B (base, chloride, sulfate); crystal violet (chloride); Victoria blue (chloride); night blue (chloride); aniline blue (alcoholic solution) (chloride); setocyanine (chloride); unnamed dye (chloride); acriflavine (chloride);

aurine; Bindschedler's green (chloride); brilliant cresyl blue (chloride); phenosafranine (chloride); methylene blue (chloride); neutral red; Meldola's blue (chloride); phenyl-*p*-phenylene diamine (chloride); tetraethyl-*p*-diamino-*p*-sulfonate triphenyl carbinol; acid fuchsin; erioglaucine A; eriogreen SFL; Helvetia blue; methyl blue (water solution); eriochrome azurol; eriochrome cyanine R; phenolphthalein (also sodium salt); *p*-amino-benzyl alcohol; aniline hydrochloride; dimethyl-*p*-toluidine; leuco-malachite green.

The method used was as follows: 1 ml. of solution was added to 1 ml. of spore suspension; the mixture was shaken and pipetted on to glass slides, five drops to a slide; slides were placed in damp chambers; spores were incubated at 25° C. for 24 hr. and examined for germination. Concentrations used were 1, 5, 10, 50, 100, 500, and 1000 parts per million. The following observations were made: all toxic dyes stained the spores and no non-toxic dyes did so; some acid dyes stimulated growth; *p*-amino derivatives of di- and triphenyl carbinol inhibited growth and the efficiency increased with the number of amino groups; when alkyl groups of increasing size were substituted in the amino group of a series of dyes, toxicity decreased with the size of the alkyl group; toxicity of malachite green dye base was reduced by substitution of ethyl, propyl, or butyl groups for methyl groups; reduction of malachite green to the leuco base removed toxicity; toxicity of basic dyes was unaffected by the acid radicle associated with the dye base; substitution of amino groups and alkylated amino groups in benzene nuclei of triphenyl methane increased toxicity, whereas acid groups reduced it; sulfonation and carboxylation reduced toxicity greatly; alkylation of amino groups increased toxicity, but alkylation of benzene nuclei had little effect; toxicity is not a function of any one part of the dye molecule but is due to the cumulative effect of the whole molecule; the toxic effect of dyes is probably due to their action on the enzyme system in the spores.—*H. P. Riley.*

KING, JOHN A. **Diaryloxylalkane derivatives.** Diphenoxyethanesulfonamides. *J. Amer. Chem. Soc.*, **66**, 2076. 1944.

A series of seventeen N-substituted α,β-diphenoxyethane-4,4′-disulfonamides and a series of ten other derivatives of α,β-diphenoxyethane have been prepared. Preliminary tests carried out with these compounds indicate that they have no trypanocidal activity.—*A. P. Bradshaw.*

MENTEN, MAUD L., JUNGE, JOSEPHINE, and GREEN, MARY H. **Distribution of alkaline phosphatase in kidney following the use of histochemical azo dye test.** *Proc. Soc. Exp. Biol. & Med.*, **57**, 82–6. 1944.

Data concerning the distribution of alkaline phosphatase in the kidney in substantial agreement with that obtained with the silver technic were obtained by direct coupling of calcium beta-naphthol with a diazotized amine. An insoluble reddish purple dye is formed. Renal phosphatase releases the calcium compound from its phosphate.—*M. S. Marshall.*

PASCHKIS, K. E., CANTAROW, A., HART, W. M., and RAKOFF, A. E. **Inhibitory action of thiouracil, thiocarbamide, and other compounds on melanin formation by tyrosinase.** *Proc. Soc. Exp. Biol. & Med.*, **57**, 37–9. 1944.

In a system containing tyrosine and tyrosinase the formation of melanin was best inhibited by thiouracil. Inhibition also occurred with thiocarbamide, glutathione, cystein, ascorbic acid, p-aminobenzoic acid, sodium sulfathiazole, and sulfadiazine. Copper and iodoacetic acid overcame the inhibition. Oxidation of tyrosine to dopa and hallachrome with subsequent inhibition of melanin formation was not disproven but direct action on tyrosinase was considered more likely.—*M. S. Marshall.*

ROHRMANN, EWALD, and SHOULE, H. A. **Derivatives of 6-methoxy-8-aminoquinoline and 2-methyl-6-methoxy-8-aminoquinoline.** I and II. *J. Amer. Chem. Soc.*, **66**, 1640–5. 1944.

Over a hundred new derivatives of 8-aminoquinolines are described. These compounds were tested for antimalarial action against *Plasmodium lophurae* in ducklings by Mr. C. L. Rose of the Lillie Research Laboratories. Complete details of their pharmacological properties will be published elsewhere.—*A. P. Bradshaw.*

TARBELL, D. S., and VAUGHN, JAMES R., JR. **Arsinimines derived from sulfanilamide. The condensation of triaryl arsines and arsine oxides with sulfanilamide and its derivatives.** *J. Amer. Chem. Soc.*, **67**, 41. 1945.

In view of the possible bacteriological importance of arsinimines derived from sulfanilamide, several such compounds were prepared. Results from tests for both arsenic and sulfanilamide type effects will be reported elsewhere.—*A. P. Bradshaw.*

WEIJLARD, JOHN, TISCHLER, MAX, and ERICKSON, A. E. **Sulfaquinoxaline and some related compounds.** *J. Amer. Chem. Soc.*, **66**, 1957. 1944.

The synthesis of 2-aminoquinoxaline as well as its conversion to 2-sulfanilamidoquinoxaline is described.

Preliminary chemotherapeutic studies indicate that 2-sulfanilamidoquinoxaline is very effective in bacterial infections and that it has an unusual property of being eliminated by the animal very slowly so that the effective concentrations can be maintained by administering it at comparatively infrequent intervals.—*Author's Summary.*

ANIMAL MICROTECHNIC

BARRETO, J. F. **Nova modificaçao ao método de Mann.** *Bol. Soc. Med. Vet. Janiero.*, **13**, No. 1. 1944.

Nervous tissue, preferably from the Ammon's horn, intended for demonstration of Negri bodies, is fixed in 10% formalin, Bouin's or Zenker's fluid, or otherwise, and stained by the following technic: (1) Remove paraffin from sections and run down to water. (2) Stain in Weigert's or Ehrlich's hematoxylin; wash in water; differentiate in HCl-alcohol; wash in water until sections are blue. (This step may be omitted if time is pressing). (3) Cover with a few drops of Mann's staining fluid (1% aqueous methyl blue, 0.7–1 ml.; 1% aqueous eosin, 1 ml.; distilled water, 6 ml.) and let stand 15 min. (4) Wash rapidly in distilled water. (5) Differentiate in picro-acetic-acid (acetic acid, strength not given, 3 drops; saturated aqueous picric acid, 15 drops; 90% alcohol, 50 ml.). Time of differentiation varies from 2 to 5 min.; sections should not be completely green. (6) Pass to 90% alcohol. (7) Dehydrate, clear in xylene, and mount in balsam.

This modification, differing from the standard Mann technic in the use of the picro-acetic differentiator, is claimed by the author to be an improvement over the original, giving the following results: cytoplasm of nerve cells, light green; nuclei and nucleoli, blue; Negri bodies, red.—*H. J. Conn.*

DUBLIN, WILLIAM B. **Combined Bodian and Masson staining method applied to skin.** *Arch. Dermat. & Syph.*, **50**, 361–2. 1944.

The preparation of sections of skin with Bodian's protargol method, counterstained with Goldner's tetrachrome modification of Masson's trichrome method or its equivalent, yields interesting results, which make the combined method of practical value on special occasions. The ingredients of the counterstain may be varied to suit the individual; Mallory's aniline-blue-orange-G may be used, but Masson's method with light green is preferred.

The following procedure proves satisfactory: (1) Fix tissue in 40% solution of formaldehyde U.S.P. diluted 1:10. Embed in paraffin, and cut sections at 6–8 μ. Remove paraffin with xylene, and hydrate in the usual manner. (2) Place sections in 1% aqueous protargol (dissolved by sprinkling on the surface of the water) for 2–4 hr. (The time can be varied; too long immersion will produce too dark an effect on epithelial and connective tissue. For skin, a surprisingly short time will suffice.) (3) Rinse briefly in tap water. (4) Reduce 5 min. in 1% aqueous hydroquinone, which may be used repeatedly, until it loses its apparent strength of action. (Other reducing agents, including photographic developing solution, may be used.) (5) Rinse briefly in tap water. (6) Tone in 0.5% aqueous gold chloride until sections turn gray. (This solution may be used until it loses its strength. The use of a metal slide tray will cause a muddy precipitate.) (7) Rinse briefly in tap water. (8) Place in 2% aqueous oxalic acid for 5 min. or less, as necessary to turn sections brownish purple. (The solution may be used as long as effective.) (9) Rinse thoroughly in running tap water. (10) Fix for 5 min. in

5% aqueous $Na_2S_2O_3 \cdot 5H_2O$ ("hypo"). Change the solution about as often as the oxalic acid. (11) Wash 5 min. in running tap water. (12) Stain 5 min. in a mixture of: 3 parts 1% "xylidine ponceau", 1 part 0.5% azophloxine, and acetic acid to make 0.1% in the whole mixture. (If ingredients are not easily available or if taste suggests, 1% acid fuchsin may be used here.) (13) Rinse briefly in 0.1% acetic acid. (14) Stain 5 min. in an aqueous solution containing phosphotungstic acid, 1.5%, orange G, 1.0%, and acetic acid, 0.1%. (15) Rinse briefly in 0.1% acetic acid. (16) Stain 2–5 min. in 0.02% aqueous light green (Masson) with addition of 0.1% acetic acid. (17) Rinse briefly in 0.1% acetic acid. (18) Pass quickly through 85 and 95% alcohol and dehydrate 8–10 min. in acetone or absolute alcohol. Clear in xylene and mount in balsam.—*H. J. Conn.*

GOVAN, A. D. TELFORD. **Experiments with Nile blue sulphate: its use in demonstrating tract degeneration in the cord.** *J. Path. & Bact.*, **56**, 447–9. 1944.

Solubility tests showed that commercial Nile blue sulfate is composed of two dyes: a chloroform-soluble red dye which resembles the Sudan fat dyes, and a water-soluble blue dye devoid of specific fat-staining properties but possessing marked adsorbability on alumina and tissues. These findings were applied to demonstrate tract degeneration in the spinal cord, with results more specific than those obtained by the Marchi or the Kulschitsky-Pal methods. The procedure is as follows: Add 0.2 g. of Nile blue sulfate to 100 ml. of distilled water and filter. Stain frozen sections for 1 min. in this solution. Wash with water. Differentiate in 1% acetic acid. This step should be prolonged; tissue cannot be over-differentiated. The blue dye is taken up by normal myelin. Degenerate myelin is rather gray, "due not so much to staining as its density of refractility".—*S. H. Hutner.*

HETHERINGTON, DUNCAN C. **Effect of trypan blue upon cardiac explants in tissue culture.** *Proc. Soc. Exp. Biol. & Med.*, **57**, 194–6. 1944.

"Cardiac explants from ten-day chick embryos, planted in a medium of lyophiled chicken plasma and embryo juice containing 1/4000 trypan blue, grew significantly better than control cultures from the same hearts grown in an identical medium without the dye". An increase in oxygen uptake is suggested tentatively as an explanation.—*M. S. Marshall.*

LENDRUM, A. **The staining of eosinophil polymorphs and enterochromaffin cells in histological sections.** *J. Path. & Bact.*, **56**, 441. 1944.

The following procedure succeeds in staining the granules of eosinophils without staining erythrocytes, thus enabling eosinophils to be counted in solid cellular tissue: Melt 1 g. phenol crystals. Add 0.5 g. chromotrope 2R (C.I. 29, Revector brand). Dissolve the sludge in 100 ml. of distilled water. The solution is ready for use and keeps well. Stain sections as usual in hemalum and counterstain in carbol-chromotrope ½ hr. Longer staining is unnecessary. Rinse and dehydrate as usual.

In human intestine, the granules of the Paneth cells are rust-colored and ill-defined. The enterochromaffin cells in human intestine fixed by formalin or formol-sublimate have fine granules stained dark brown, showing up these cells very distinctly. The color is a redder brown in tissues fixed in chromate solutions.—S. *H. Hutner.*

LILLIE, R. D. **Factors influencing the Romanowsky staining of blood films, and the role of methylene violet.** *J. Lab. & Clin. Med.*, **29**, 1181–97. 1944.

The presence of more than 4% of methylene violet is tolerated in Romanowsky staining only in "on slide" technic using relatively high concentrations of methanol in the final staining mixture. More than 4% will cause precipitation of both the methylene violet and the thiazin eosinates. An initial concentration of 25–33% methanol seems the optimum for on slide staining. Alkali polychroming methods are difficult to control so as to produce constant proportions of the various azures, and they also produce a variable amount of methylene violet. Acid chromate polychroming is easily controlled, yields relatively constant products, and produces little or no methylene violet. It permits the use of

commercial double $ZnCl_2$ as well as medicinal methylene blue. Acid chromate polychroming with 200 mg. $K_2Cr_2O_7$ per gram of medicinal methylene blue yields chiefly azure B, with some methylene blue. A 1:24 dilution of 1% solution of this eosinate in equal parts of methanol and glycerol is recommended for staining both blood and parasites.—*John T. Myers.*

LILLIE, R D. **Myelin staining by a fixed schedule for the occasional user.** *Arch. Path.*, **37**, 392. 1944.

Material fixed in dilute aqueous formaldehyde solution and not over 6 months old should be mordanted 2–4 days in 5% $K_2Cr_2O_7$ before dehydration. Chromation of paraffin sections of formaldehyde-fixed tissue is worthless. The mordanted deparaffinized sections are then stained as follows: Stain 40 min. at 55° C. in iron alum hematoxylin (1:1 mixture of 1–5 day old 1% alcoholic hematoxylin and 4% $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$); wash in water, and differentiate 1 hour in 0.5% iron alum; wash in water and treat 10 min. with a solution containing 1% sodium tetraborate and 2.5% potassium ferricyanide; wash in water and counterstain 5 min. in 0.1% safranin O (certification lot No. NS-11) in 1% acetic acid; wash in water; dehydrate, clear, and mount, using a solution order of acetone, acetone plus xylene, xylene (2 changes) and 60% xylene-clarite.—*L. Farber.*

RANDOLPH, T. G. **Differentiation of leucocytes in the counting chamber by propylene glycol-aqueous stains: A screen for the detection of major blood abnormality.** *Amer. J. Clin. Path., Tech. Sect.*, **8**, 48. 1944.

A white blood cell diluting fluid is prepared by mixing equal amounts of 0.05% methylene blue in 50% propylene glycol and 0.05% phloxine in 50% propylene glycol. This solution remains usable for 4 hr. A period of 10–15 min. is recommended after the addition of diluting fluid before counts are made. With this fluid the eosinophile granules appear red and the nuclei of all leucocytes green blue. Possible interpretations of abnormal white cell counts are given.—*L. Farber.*

MICROÖRGANISMS

BARTHOLOMEW, J. W. and UMBREIT, W. W. **Ribonucleic acid and the Gram stain.** *J. Bact.*, **48**, 567–78. 1944.

Removal of ribonucleic acid from several kinds of Gram-positive organisms left them Gram-negative. Either choleate or ribonuclease removed the ribonucleic acid from killed yeast or bacteria. The Gram-negative organisms were smaller than the corresponding Gram-positive ones. It was possible to "replate" ribonucleic acid on the organisms from which it had been removed, either with the material extracted from them or with yeast ribonucleic acid (Eastman) under certain conditions. They were then again Gram-positive and their original size. It was not possible by the same method to add ribonucleic acid to organisms that were originally Gram-negative. Theories of Gram staining are discussed on the basis of these observations.—*V. Kavanagh.*

FREIMAN, D. G. and MOKOTOFF, G. F. **Demonstration of tubercle bacilli by fluorescence microscopy. A comparative evaluation of the fluorescence and Ziehl-Neelsen methods in the routine examination of smears for acid-fast bacilli.** *Amer. Rev. Tuberc.*, **46**, 434–42. 1943.

The sensitivity of the fluorescence method in the examination of sputum and gastric smears for acid-fast bacilli was investigated by comparing its performance with the Cooper modification of the Ziehl-Neelsen method. A significant increase (93%) in the number of positive specimens found by fluorescence was obtained only in the group of gastric concentrates. Differences observed in the examination of untreated and concentrated sputum specimens were too small to be significant and in the latter case were less than the differences obtained when a series of duplicate smears were examined by the Ziehl-Neelsen method alone. The fluorescence method appears to offer little advantage over the less exacting Ziehl-Neelsen method, especially in view of the fact that routine

culture methods used in this laboratory are considerably more sensitive than either.—*Author abstract.* (Courtesy *Biological Abstracts*).

GRAHAM, CLARENCE F. **Fluorescence microscopy of Mycobacterium tuberculosis. A study of basic factors.** *Amer. Rev. Tuberc.*, **48**, 421–34. 1943.

The author has investigated the basic factors of the technic as employed at present. The efficiency of the various dyes and filters as well as sources of light, were determined by spectrographic analysis. No satisfactory glass filter has thus far been produced. Copper ammonium sulfate solution is the best substitute. No satisfactory light source has been found, but a 500-watt projection bulb with condensing lenses gives the best results. Real evaluation of the method depends upon the availability of improved apparatus.—*L. Thompson.* (Courtesy *Biological Abstracts*).

TOMLINSON, W. J., and GROCOTT, R. G. **A simple method of staining malaria protozoa and other parasites in paraffin sections.** *Amer. J. Clin. Path.*, **14**, 36. 1944.

A staining procedure for tissue sections is described which clearly shows up malaria and other protozoal organisms as well as other parasitic forms.

Fixed, embedded and deparaffined sections are treated as follows: Stain 5 min. in phloxine solution (1 g. National Aniline phloxine, certification No. NPh-9, C.I.778, dissolved in 98 ml. distilled water to which 1 ml. of 1% $CaCl_2$ has been added); rinse 1 min. in tap water; stain, with gentle agitation, 45 sec. to 1 min. in toluidine blue solution (1 g. National Aniline toluidine blue, cert. No. NU-2, C.I.925, dissolved in 75 ml. distilled water containing 0.5 g. Li_2CO_3, and let stand 3–4 days at room temp. or 24 hr. at 37° C., return volume to 75 ml., add 20 ml. glycerol and 5 ml. absolute ethanol); rinse 1 min. in tap water and drain; immerse 2 min. in acetone; transfer to 0.2% by vol. acetic acid (made fresh daily), and agitate gently until clouds of blue no longer appear; place 2 min. in acetone. Differentiate 1–2 min. in solution composed of 75 ml. of 5% solution of colophony (rosin yellow) in acetone, 25 ml. of absolute ethanol and 0.5 to 1 ml. of 1% orange G, National Aniline, cert. No. NO-8, C.I.27, prepared fresh daily. Treat 3 min. with acetone, then 3 min. each with 2 changes of 1:1 acetone-xylene, 3 changes of xylene each for 5 min., mount in a neutral medium such as synthetic resin like Permount.

To remove the malaria pigment from tissues, the sections after deparaffination are placed in 5% U.S.P. ammonium sulfide solution (U.S.P. solution diluted once with distilled water) for 2–24 hours, depending on the tissue and degree of pigmentation, and then washed in tap water for 15 min. until no more typical odor is present. The sections are then stained as described above.

With this procedure, tissue nuclei stain blue to purple with lavender nucleoli, the cytoplasm pale red with deep red granules, the erythrocytes red to orange red, the human malaria parasites pale blue with sharp margins within the erythrocytes, and the nuclear chromatin in trophozoites deep purple to lavender rose. The leishimanian forms of *T. cruzi* and *L. tropica* show red-purple nuclear chromatin, deep blue-black kinetoplast and pale blue cytoplasm, with a prominent border producing a halo effect. Microfilaria, various stages of *Endamoeba histolytica* and *E. coli* and *Balantidium coli* are clearly recognizable.—*L. Farber.*

MEDALIS, L. S., KAHANER, J. R., and SINGER, A. J. **Staining method for thin and thick smears for malaria and other cell-containing body fluids.** *Amer. J. Clin. Path., Tech. Sect.*, **8**, 68. 1944.

Thick and thin blood films on the same slide are stained as follows: Fix the thin portion of the smear by dipping 1 min. in absolute methanol, taking care not to touch the thick smear with the methanol. Rinse entire slide gently in water, holding the thick smear uppermost, and immerse slide 4 sec. in acid eosin solution (0.2% eosin Y in 0.56% Na_2HPO_4 and 1.70% KH_2PO_4). Rinse in running water and dip for 6 sec. in alkaline methylene blue solution (0.16% methylene blue, 0.10% azure II in a solution of 0.56% KH_2PO_4 and 1.70% Na_2HPO_4). Rinse in water and dry in air.

For smears of cell-containing body fluids (including blood), the procedure is as follows: Prepare smears by placing a drop of sediment or small portion of pellicle on a slide, cover with another one, and slide the two apart. Fix by dipping

in methanol and wash in cold water. Dip 4 sec. in above acid eosin solution, wash, and treat with above alkaline methylene blue solution for 6 sec. Wash in water and dry in air.

The appearance of the cellular elements in smears stained in this manner is similar to that observed with Wright's staining technic.—*L. Farber.*

MULLER, H. **On the use of a modification of Pryce's slide culture method for the estimation of the bacteriostatic power of chemicals on the tubercle bacillus.** *J. Path. & Bact.*, **56**, 429–33. 1944.

By observation of the growth of tubercle bacilli in colonies on micro-slides, a convenient method is available for carrying out comparative tests of the action of bacteriostatic agents under uniform conditions. The procedure is as follows: Cut "chip" slides ¾ x ¼ in. from ordinary slides (16 "chip" slides may be cut from one 3 x 1 in. slide). Scratch a number on the opposite side to identify the culture. Spread sputum on the same side as the number, but covering only ¼ of the opposite end. To spread the sputum evenly (important), dilute a fresh strongly positive sputum with an equal volume of water and shake thoroughly with a few glass beads. (As an alternate procedure, a colony on artificial medium may be ground between slides with the gradual addition of water until a thick suspension is obtained.) Centrifuge and pipette off the supernatant. Mix with an equal volume of fresh serum. Use a small standard wire loop to place an equal volume on each slide. After drying, place the slides in 10% H_2SO_4 for 10 min., film side up. Wash in 2 changes of sterile water in Petri dishes, and place at the bottom of the culture bottle (a ¼ oz. screw-capped bottle). After 1 week's incubation remove the slides, wash gently in water, dry, and stain by immersion in a Petri dish of warm carbol fuchsin. After differentiation, wash and dry and mount in Canada balsam on an ordinary slide, sputum film upwards. The sputum ends are covered by a cover slip, leaving the identification numbers exposed. The colonies are then compared.

The routine culture medium consists of equal parts of the corpuscles of citrated blood in twice their volume of distilled water. This medium yields large colonies in a week.

Potassium tellurite was the most bacteriostatic of the substances tested. The tests included a number of sulfonamide drugs.—*S. H. Hutner.*

RANDOLPH, T. G., and MIKELE, R. F. **Carbol fuchsin in propylene glycol for rapid staining of the tubercle bacillus. Preliminary report.** *Amer. Rev. Tuberc.*, **49**, 109. 1944.

Optimum staining qualities result from the use of stock solutions prepared as follows: Solution A, 1% basic fuchsin in propylene glycol; Solution B, 5% phenol in distilled water. Mix solution A with solution B in proportion of 1:4, shake vigorously until uniformly mixed. The mixture is stable at room temperature at least 2 months.

Slides are stained as follows: Immerse or flood slide 4 min. or longer in above mixture; wash with tap water; decolorize 45–60 sec. in acid alcohol; counterstain 45–60 sec. with either methylene blue or brilliant green (concentrations not given). The organisms stain a bright red and are easily recognized against the background of the counterstain.—*L. Farber.*

SMITH, N. R., and DAWSON, VIRGINIA T. **The bacteriostatic action of rose Bengal in media used for plate counts of soil fungi.** *Soil Sci.*, **58**, 467–71. 1944.

The authors suggest the use of a 1–15,000 dilution of rose Bengal in agar media, to inhibit bacteria when plate counts of fungi are wanted. It inhibits nearly all soil bacteria, while those that do develop produce soft, glistening colonies that cannot be confused with fungi. The medium of choice contains only glucose, nitrate, soil extract, and agar, and has the advantage of repressing the fungi that produce spreading colonies and of preventing practically all bacterial growth. A peptone agar may be used, but does not seem to be quite so selective for fungi. The authors find rose Bengal to be more efficient than an acid reaction (pH 4.2) in repressing bacteria and to be superior to other inhibiting agents such as boric acid, benzoic acid, or chrysoidine Y.—*H. J. Conn.*

SMYTH, J. D. **A technique for mounting free-living protozoa.** *Science*, **100**, 62. 1944.

A method for mounting free-living protozoa is described which overcomes many of the usual difficulties related to extensive centrifuging. The organisms are fixed in Schaudinn's fluid and brought through 70% and 90% into absolute alcohol by gentle centrifuging. A small drop of egg albumen is placed on a clean slide and a very thin film produced by smearing it with the edge of another slide—as in the preparation of a blood film. A drop of the concentrated organisms is allowed to fall on the film of albumin from a fine pipette held about an inch above the slide. The combined action of the dropping force and the rapid coagulation of the albumen by the alcohol, immediately causes the organisms to be fixed securely to the slide. The slides are then placed in absolute alcohol and treated as ordinary sections. The film of albumen is so thin that it causes no interference with the staining reactions of the protozoa.—*T. M. McMillion.*

STEINER, GABRIEL and STEINER, GRETE. **New simple silver stain for demonstration of bacteria, spirochetes and fungi in sections from paraffin embedded tissue blocks.** *J. Lab. & Clin. Med.*, **29**, 868–71. 1944.

The following method does not require frozen sections or the application of heat: Remove paraffin from sections in the usual manner; place them in 1% aqueous $UO_2(NO_3)_2$ for 3 min.; wash in distilled water; immerse in 1% aqueous $AgNO_3$ for 2 hr. in an oven at 56°–58° C.; wash thoroughly with distilled water; dehydrate up to absolute alcohol; place in absolute alcohol containing 2.5% gum mastic for 5 min.; transfer without washing into the reducing solution (1 g. hydroquinone in 60 ml. of distilled water to which is added 20 ml. of 2.5% gum mastic in absolute alcohol and 20 ml. of solution obtained by dissolving 2 g. $AgNO_3$ in 1000 ml. of boiling distilled water, adding 1.65 g. of powdered sodium potassium tartrate, and filtering) for 12–15 min., or until the sections become light brown; wash 3 times in distilled water; dehydrate; clear in xylene and mount in Canada balsam.—*John T. Myers.*

YEGIAN, D., and PORTER, K. R. **Some artifacts encountered in stained preparations of tubercle bacilli. I. Non-acid-fast forms arising from mechanical treatment.** *J. Bact.*, **48**, 83–91. 1944.

No non-acid-fast fine rods or granules appeared in slides of the surface film of young cultures unless these films had been smeared, or sectioned after fixation, dehydration, and embedding. The number of such rods and granules increased with an increase in the time or intensity of smearing of fresh films, or with the dullness of the microtome knife or the thinness of the section.— *Virgene Kavanagh.*